ANTIBIOTIC PRODUCTION (II)

Reference to Provisional Application

5 This application claims the benefit of U.S. Provisional

Application No. 60/242,533 filed on October 23, 2000, the

entire disclosure of which is incorporated by reference

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herein.

Introduction

Malonyl-CoA is essential as a metabolic substrate in all

15 living organisms studied so far and it also plays a role as

a modulator of specific protein activity (for a review see

Brownsey *et al.,* 1997). Malonyl-CoA is a substrate for

fatty acid synthase (FAS) (Bloch and Vance, 1977), for

polyketide synthases (PKS) in plants, fungi and bacteria

20 (Hopwood & Sherman, 1990) and for fatty acid chain-

elongation systems (Saggerson, *et al.,* 1992). Understanding

the pathway(s) that lead to the biosynthesis of malonyl-CoA

in *Streptomyces* might have an outstanding interest, since

these micro-organisms are well known to have the ability to

25 synthesize a vast array of pharmaceutically important

polyketide compounds (such as antibiotic, antiparasitic,

antifungal, immunosuppressant and/or antitumour

polyketides), where malonyl-CoA is used as the most common

extender unit (Hopwood & Sherman, 1990). Therefore,

30 information gained on the enzyme(s) involved in the supply

of this key metabolite will be relevant, not only for a

better understanding of the primary metabolism of

Streptomyces, but for improving production of many useful

secondary metabolites.

35 Biosynthesis of malonyl-CoA occurs in most species through

the ATP-dependent carboxylation of acetyl-CoA by an acetyl-

CoA carboxylase (ACCase) (Bloch & Vance, 1977; Harwood,

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1988). The overall reaction catalyzed by ACCase is a two step process that involves ATP-dependent formation of carboxybiotin followed by transfer of the carboxyl moiety to acetyl-CoA. The importance of this biosynthetic pathway

5   is most directly reflected by the fact that ACCase expression is essential for normal growth of bacteria (Perez, et al., 1998; Li and Cronan, 1993), yeast (Hasselacher, et al., 1993) and isolated animal cells in culture (Pizer, et al., 1996).

10   Several complexes with ACCase activity have been purified from various actinomycetes. Interestingly, these complexes have also shown the ability to carboxylate other substrates like propionyl- and butyryl-CoA (Erfle, 1973; Henrikson and Allen, 1979; Huanaiti and Kolattukudy, 1982). This

15   property has led to these enzyme being called acyl-CoA carboxylases, and all of them have been shown to consist of two subunits, a larger one (α-chain) with the ability to carboxylate its covalently bound biotin group, and a smaller sub-unit (β-chain) bearing the carboxyl transferase

20   activity. However, there is no information gained, so far, regarding the physiological role of these enzymes.

In *Streptomyces* the purification of a complex with ACCase activity has proved to be unsuccessful, probably due to its high instability (Bramwell et al., 1996). However ACCase

25   activity has been readily measured in crude extracts of *S. coelicolor* (Bramwell et al., 1996; Rodríguez and Gramajo, 1999), indicating that this enzyme complex was present in this micro-organism.

A pathway for the biosynthesis of malonyl-CoA in *S.*

30   *aureofaciens* has been described that does not involve ACCase (Behal et al., 1977; Laakel et al., 1994). This route involves the anaplerotic enzymes phosphoenolpyruvate

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carboxylase and oxaloacetate dehydrogenase. In *S. coelicolor* A3(2), no evidence for the presence of oxaloacetate dehydrogenase has been found (Bramwell *et al.*, 1993); thus, biosynthesis of malonyl-CoA in this organism

5   seemed to occur exclusively through the ACCase enzyme activity.

Attempts carried on in *S. coelicolor* to characterize enzymes with carboxylase activity, have led to the characterization of two complexes exhibiting exclusively

10 PCCase activity. The PCCase purified by Bramwell *et al.*, (1996) comprises a biotinylated protein of 88 kDa, PccA, and a non-biotinylated component, the carboxyl transferase, of 66 kDa. More recently the inventors have also characterized at both the genetic and biochemical levels,

15 the components of a second PCCase in this bacterium. *In vitro* reconstitution experiments have shown that an active complex could be obtained by mixing a carboxyl transferase component of 59 kDa (deduced MW, though it runs anomalously in SDS-PAGE, with an apparent MW of 65 kDa), PccB, with

20 either of the two almost identical biotinylated components named AccA1 and AccA2 (Rodríguez and Gramajo, 1999).

Recently a gene cluster encoding malonyl-CoA decarboxylase (MatA), malonyl-CoA synthetase (MatB) and a putative decarboxylate carrier protein (MatC) has been proposed as

25 the pathway for malonate metabolism in *Rhizobium trifolii* (An and Kim, 1998). After the transport of the malonate by MatC, the malonate is converted into malonyl-CoA by MatB and finally decarboxylated to acetyl-CoA by MatA. However, the fact that the K_m of the malonyl-CoA decarboxylase for

30 malonyl-CoA is relatively high has led the inventors to propose that malonyl-CoA synthesised from malonate by malonyl-CoA synthetase (rather than malonyl CoA synthesised by ACCase) is the major source for fatty acid biosynthesis

in the bacterioid *R. trifolii*. Interestingly, genes with very high identity to MatC and MatB have been recently reported in the *S. coelicolor* genome project, suggesting that malonyl-CoA could also be synthesized from malonate in

5   this micro-organism.

The inventors have identified an essential acyl-CoA carboxylase of *S. coelicolor*, and provide detailed genetic and biochemical characterization. The enzyme complex

10   contains a unique sub-unit composition and appears to be the main pathway for the biosynthesis of malonyl-CoA, one of the key metabolites in the linkage between primary and secondary metabolism. An alternative pathway for the biosynthesis of malonyl-CoA is also proposed for cultures

15   growing in malonate, and it most probably involves the *matB* and *matC* homologues of *R. trifolii*. However, even in these growing conditions, the acyl-CoA carboxylase seems to be essential for the viability of the micro-organism.

20   **Summary of invention**

Two genes *accB* and *accE*, forming a single operon, have been cloned from *Streptomyces coelicolor* A(3)2. The deduced amino acid sequence of AccB showed high similarity to

25   carboxyl transferase of several propionyl- or acyl-CoA carboxylases of different actinomycetes. By contrast, AccE did not show any significant homology with protein sequences deposited in the GenBank data base. Heterologous expression of *accB* and *accE* in *Escherichia coli* and *in*

30   *vitro* reconstitution of enzyme activity in the presence of the biotinylated component AccA1 or AccA2 confirmed that AccB was the carboxyl transferase subunit of an acyl-CoA carboxylase.

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These experiments also established that AccE was a necessary component to obtain a fully active enzyme complex, whose subunit composition seems to be unique

5   within this type of carboxylase. Gene disruption experiments clearly determined that AccB was essential for *S. coelicolor* viability. This protein together with AccA2, a biotinylated component essential for the viability of this micro-organism (Rodríguez and Gramajo, 1999), are the

10   best candidates to form an acyl-CoA carboxylase, whose main physiological role is, most probably, the biosynthesis of malonyl-CoA.

Transcriptional studies of *accBE*, *accA2* and *accA1* have

15   shown that *accBE* and *accA2* are mainly expressed during vegetative and transition phase of growth, although some expression of these genes also occurred during stationary phase where they should provide the malonyl-CoA subunits for secondary metabolites biosynthesis. *accA1* is only

20   expressed during the transition phase of growth and its role in the formation of a carboxylase complex involved in providing the substrate for polyketide compounds of *S. coelicolor* is discussed.

25   Finally, an alternative route for the biosynthesis of malonyl-CoA is proposed when malonate is used as a carbon source. However, this route seems unable to substitute the main one, determined by the acyl-CoA carboxylase.

30   Accordingly, in a first aspect, the present invention provides a nucleic acid comprising a nucleic acid sequence which encodes an AccB polypeptide and/or an AccE polypeptide, or a nucleic acid sequence complementary thereto.

In a second aspect, the present invention provides a
nucleic acid comprising a nucleic acid sequence which
encodes an AccA1 and/or AccA2 polypeptide, or a nucleic
5   acid sequence complementary thereto. It is believed that
such nucleic acid was not made available to the public
before 24 October 1999, when the amino acid sequences of
these polypeptides were disclosed in an oral presentation.

10 Exemplary nucleic acid sequences encoding the AccB, AccE,
AccA1 and AccA2 polypeptides are given herein. Preferred
embodiments of the invention include such sequences.
However, it would be a matter of routine for the skilled
person to obtain other nucleic acid sequences encoding
15 these polypeptides, e.g. by introducing mutations which do
not alter the encoded amino acid sequence, by virtue of the
degeneracy of the genetic code, or by introducing mutations
which alter the encoded amino acid sequence, within limits
as defined below. Moreover, nucleic acids encoding
20 variants of the polypeptides may be obtained e.g. by
screening different strains of *S. coelicolor* or closely
related species of *Streptomyces* using degenerate probes
based on the sequences given herein.

25 Preferred nucleic acids of the first and second aspects
encode AccB and AccE polypeptides along with an AccA1
and/or an AccA2 polypeptide (preferably AccA2).

The nucleic acid sequences encoding Acc polypeptides are
30 preferably in operative association with regulatory
sequences, e.g. sequences which enable constitutive or
inducible expression in *Streptomyces* species. Examples of
plasmids which include such regulatory sequences and of
suitable promoters are given herein. A suitable inducible

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promoter is tipA (inducible by thiostrepton); suitable
constitutive promoters are ermE and the optimised ermE*.
Alternatively, naturally occurring nucleic acid sequences
may be in operative association with the regulatory

5   sequences with which they are normally associated, or
corresponding regulatory sequences from homologous genes in
other strains or species. For example, the nucleic acid
sequences may be in operative association with the
corresponding regulatory (e.g. promoter) sequences defined

10   herein.

For detailed protocols relevant to this and other aspects,
see standard reference texts, such as Sambrook et al.
(1989) and Hopwood et al. (1985).

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In a third aspect, the present invention separately
provides AccB, AccE, AccA1 and AccA2 polypeptides having
amino acid sequences encoded or encodable by the respective
nucleic acid sequences referred to in the first and second

20   aspects.

In a fourth aspect, the present invention provides: vectors
containing the nucleic acids of the first and second
aspects (preferably vectors, e.g. plasmids, suitable for

25   transforming *Streptomyces* species for expression therein)
and cells, particularly *Streptomyces* cells, transformed
with such vectors. Furthermore, the present invention
provides a method of producing a secondary metabolite of a
Streptomyces species, the method comprising culturing such

30   transformed *Streptomyces* cells and extracting the secondary
metabolite from the cell culture. The metabolite may be
purified and/or formulated as a commercial product
according to standard procedures.

In a fifth aspect, the invention provides a method of modifying a secondary metabolite-producing strain of a *Streptomyces* species to increase production of said secondary metabolite, the method comprising modifying said

5   strain to express, or to increase expression of, nucleic acid encoding one or more polypeptides selected from the group consisting of AccB, AccE, AccA1 and AccA2.

In a sixth aspect, the present invention provides a method

10   of modifying a strain of a *Streptomyces* species to increase ACCase and/or PCCase activity, the method comprising modifying said strain to express, or to increase expression of, nucleic acid encoding one or more polypeptides selected from the group consisting of AccB, AccE, AccA1 and AccA2.

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In a seventh aspect, the present invention provides a modified strain of a *Streptomyces* species, produced or producible according to the method of the fifth or sixth aspect. Also provided are cells of said strain, methods of

20   producing secondary metabolites comprising culturing said cells and extracting the secondary metabolite, which may be purified and/or formulated as a commercial product.

In an eighth aspect, the invention provides a method of

25   increasing production of a secondary metabolite in cells of a *Streptomyces* species, the method comprising culturing said cells in the presence of exogenous malonate, preferably at a concentration of at least about 0.1%, more preferably at least about 0.2%, 0.4%, 0.5%, 0.75% or 1%,

30   though higher concentrations may be used. 1% represents 1g per 100 ml of medium.

Detailed Description

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In relation to the fifth and sixth aspects, the modification preferably provides for increased expression of nucleic acid encoding more than one of AccB, AccE, AccA1 and AccA2, more preferably at least AccB and AccE or at

5   least AccB and either AccA1 or AccA2, more preferably AccB, AccE and either AccA1 or AccA2. Of AccA1 and AccA2, AccA2 is preferred. Increased expression of nucleic acid encoding both AccA1 and AccA2 (usually in combination with AccB and optionally AccE) is also contemplated.

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The methods of the fifth and sixth aspects preferably include a step of transforming a *Streptomyces* cell with a said nucleic acid under the control of a constitutive or inducible promoter, preferably a strong promoter. However,

15   the expression of existing said nucleic acid could be increased, e.g. by placing them under the control of a stronger promoter sequence or sequences.

Exogenous said nucleic acid can replace existing said

20   nucleic acid in the cell, or can be added without removing or functionally deleting existing said nucleic acid.

Acc polypeptides and *acc* genes

In the definitions herein of the invention, and of the

25   scope of protection (but not, except where the context requires otherwise, in the experimental sections), the term AccB is intended to include not only a polypeptide having the deduced amino acid sequence encoded by the nucleic acid sequence of Fig. 12 (though this is a preferred

30   embodiment), but also a polypeptide which is a variant (e.g. an allelic or isoallelic variant) or a derivative of said polypeptide, having at least about 60% amino acid identity with said polypeptide, preferably at least about 65%, 70% or 75%, especially preferably (in view of the

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similarity of AccB as disclosed herein to another protein
of unconfirmed function) at least about 80%, 85%, 90%, 92%,
94%, 96%, 98% or 99% identity. Such a variant or
derivative may possess any one or more of the biological

5 properties of the wild-type AccB protein, as disclosed
herein, e.g. complex formation with AccA1, AccA2 and/or
AccE (or allosteric regulation by AccE), ACCase and/or
PCCase activity when AccB is co-expressed with AccA1, AccA2
and/or AccE, or increased secondary metabolite production

10 when AccB is overexpressed in *Streptomyces* species
(preferably in conjuction with AccA1, AccA2 and/or AccE).

Similarly, the term AccE is intended to include not only a
polypeptide having the deduced amino acid sequence encoded

15 by the nucleic acid sequence of Fig. 13 (though this is a
preferred embodiment), but also a polypeptide which is a
variant (e.g. an allelic or isoallelic variant) or a
derivative of said polypeptide, having at least about 40%
amino acid identity with said polypeptide, preferably at

20 least about 50%, 60%, 70%, 80%, 85%, 90%, 95% or 99%
identity. Such a variant or derivative may possess any one
or more of the biological properties of the wild-type AccE
protein, as demonstrated herein, e.g. complex formation
with AccA1, AccA2 and/or AccB (or allosteric regulation of

25 AccB), ACCase and/or PCCase activity when AccE is co-
expressed with AccB, or increased secondary metabolite
production when AccE is overexpressed in *Streptomyces*
species (preferably in conjuction with AccB).

30 Similarly, the terms AccA1 and AccA2 are intended to
include not only the polypeptides having the amino acid
sequences shown in Fig. 11 (though these are respective
preferred embodiments), but also polypeptides which are
variants (e.g. allelic or isoallelic variants) or are

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derivatives of said polypeptides, having at least about 75%
amino acid identity with said polypeptide, preferably at
least about 80%, 85%, 90%, 92%, 94%, 96%, 98% or 99%
identity. Such variants or derivatives may possess any one
5   or more of the biological properties of the wild-type AccA1
or AccA2 polypeptides, as disclosed herein, e.g. complex
formation with AccB and/or AccE, ACCase and/or PCCase
activity when AccA1 or AccA2 is co-expressed with AccB
and/or AccE, or increased secondary metabolite production
10   when AccB is overexpressed in *Streptomyces* species
(preferably in conjuction with AccB and/or AccE).

A variant or a derivative of a given peptide may have one
or more of internal deletions, internal insertions,
15   terminal truncations, terminal additions, or substitutions
of one or more amino acids, compared to the given peptide.

References to nucleic acid encoding AccA1, AccA2, AccB
and/or AccE (or to *accA1, accA2, accB* and/or *accE* genes)
20   should be interpreted accordingly.

In relation to the first aspect, preferred nucleic acids
comprise a nucleic acid sequence having at least about 50%,
preferably at least about 60%, 70%, 80%, 85%, 90%, 95%, 98%
25   or 99% nucleic acid sequence identity with the *accB* nucleic
acid sequence shown in Fig. 12. Other preferred nucleic
acids comprise a nucleic acid sequence having at least
about 40%, preferably at least about 50%, 60%, 70%, 80%,
85%, 90%, 95%, 98% or 99% nucleic acid sequence identity
30   with the *accE* nucleic acid sequence shown in Fig. 13.
Similarly, in relation to the second aspect, preferred
nucleic acids comprise a nucleic acid sequence having at
least about 50%, preferably at least about 60%, 70%, 80%,
85%, 90%, 95%, 98% or 99% nucleic acid sequence identity

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with the *accA1* or *accA2* nucleic acid sequence shown in Fig. 11.

<u>Secondary metabolites and *Streptomyces* species</u>

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While the experimental disclosure herein relates to the production of Act (actinomycin) and Red (undecylprodigiosin) in *S. coelicolor* A3(2) (strain M145), it is thought that the teaching is applicable to other

10 strains of *Streptomyces* in particular, it is thought that overexpession of all three Acc polypeptides (i.e. AccB, AccE and AccA1 and/or AccA2) will lead to increased malonyl-CoA production in substantially any *Streptomyces* species or even in other actinomycetes or in fungi (which

15 also produce polyketide compounds). Since malonyl-CoA is an essential metabolic substrate, it is thought that this will lead to greater yield of desired secondary metabolites (for which see page 1), e.g. polyketides (including antibiotic polyketidss) and fatty acids.

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Preferred secondary metabolites are, however, antibiotics, especially Act and Red.

Preferred *Streptomyces* species are the closely related

25 species *S. coelicolor*, *S. violaceoruber*, *S. lividans* and *S. parvulus*, especially *S. coelicolor*. Strains of such species are commonly available, e.g. from the ATCC, for example under ATCC deposit numbers 12434 for *S. parvulus* and 19832 for *S. violaceoruber*. *S. coelicolor* A3(2) and *S.*

30 *lividans* 66 are available from the John Innes Culture Collection (Norwich, UK) under JICC deposit numbers 1147 and 1326, respectively. However, the invention is not limited to such particular strains.

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<u>Acetyl-CoA</u>

In preferred embodiments, present invention further
provides for the increased production in *Streptomyces* of
5   acetyl-CoA, since it is thought that when ACCase activity
is increased by the methods and means of the present
invention, production of malonyl-CoA may become limited by
the availability of the substrate acetyl-CoA. It is
proposed that increased acetyl-CoA production could then
10   lead to a further increased rate of malonyl-CoA production
and hence secondary metabolite production. For example,
oils or fatty acids could be used as the carbon source
(together with glucose); fatty acids are degraded by b-
oxidation giving high levels of acetyl-CoA.
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<u>Sequence identity</u>

"Percent (%) amino acid sequence identity" is defined as
the percentage of amino acid residues in a candidate
20   sequence that are identical with the amino acid residues in
the sequence with which it is being compared, after
aligning the sequences and introducing gaps, if necessary,
to achieve the maximum percent sequence identity, and not
considering any conservative substitutions as part of the
25   sequence identity. The % identity values used herein are
generated by WU-BLAST-2 which was obtained from Altschul et
al. (1996); http://blast.wustl/edu/blast/README.html. WU-
BLAST-2 uses several search parameters, most of which are
set to the default values. The adjustable parameters are
30   set with the following values: overlap span =1, overlap
fraction = 0.125, word threshold (T) = 11. The HSPS and
HSPS2 parameters are dynamic values and are established by
the program itself depending upon the composition of the
particular sequence and composition of the particular

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database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity. A % amino acid sequence identity value is determined by the number of matching identical residues

5   divided by the total number of residues of the "longer" sequence in the aligned region, multiplied by 100. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-BLAST-2 to maximize the alignment score are ignored).

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"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the sequence under comparison. The identity values used

15   herein were generated by the BLASTN module of WU BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

Culture and Purification

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Methods of genetic manipulation, cell culture and purification of expression products produced in cell culture are well known to the skilled person, e.g. from standard textbooks such as Sambrook et al (1989). In

25   particular, methods for genetically manipulating *Streptomyces*, culturing *Streptomyces* under conditions suitable for secondary metabolite (e.g. polyketide and/or antibiotic production) and purifying secondary metabolites from *Streptomycete* cell culture medium are well known, e.g.

30   from Hopwood et al. (1985) and Kieser et al (2000).

Formulation

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Similarly, methods of formulating active compounds (e.g. polyketides, particularly antibiotics) as pharmaceuticals are well known in the art. Such pharmaceutical formulations may comprise, in addition to the active

5   compound, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other

10   material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, transdermal, transmucosal, intramuscular, intraperitoneal routes.

Suitable carriers, adjuvants, excipients, etc. can be found

15   in standard pharmaceutical texts, for example, Remington's Pharmaceutical Sciences, 18th edition, Mack Publishing Company, Easton, Pa., 1990; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

20   Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or

25   vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

30   For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active compound will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the

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art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or

5   other additives may be included, as required.

Formulations suitable for transmucosal administration include liquids, solutions, suspensions, emulsions, suppositories, pessaries, gels, pastes, ointments, creams,

10 lotions, oils, as well as patches, adhesive plasters, depots, and reservoirs.

Formulations suitable for transdermal administration include gels, pastes, ointments, creams, lotions, and oils,

15 as well as patches, adhesive plasters, bandages, dressings, depots, and reservoirs.

Ointments are typically prepared from the active compound and a paraffinic or a water-miscible ointment base.

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Creams are typically prepared from the active compound and an oil-in-water cream base. The aqueous phase of the cream base may include at least about 30% w/w of a polyhydric alcohol, i.e., an alcohol having two or more hydroxyl

25 groups such as propylene glycol, butane-1,3-diol, mannitol, sorbitol, glycerol and polyethylene glycol and mixtures thereof. The topical formulations may desirably include a compound which enhances absorption or penetration of the active compound through the skin or other affected areas.

30 Examples of such dermal penetration enhancers include dimethylsulfoxide and related analogues.

Formulations may suitably be provided as a patch, adhesive plaster, bandage, dressing, or the like which is

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impregnated with one or more active compounds and optionally one or more other pharmaceutically acceptable ingredients, including, for example, penetration, permeation, and absorption enhancers.

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences (*supra*).

A pharmaceutical formulation may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

The work underlying the invention will now be described in detail, by way of example only, with reference to the accompanying figures.

Figures

Fig. 1   Organization of the genomic region of *S. coelicolor* M145 chromosome harbouring *accB* and *accE*

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genes. A. Genetic and physical map of the 6.2 kb
insert in pRM08. The secondary structure downstream
accE represents a rho-independent transcriptional
terminator. Fragments I and II were amplified by PCR

5   with the pair of oligos accBup-accBdown and accBEup-
accBEdown respectively, uniquely labelled at the 5'-
end (*) and used as probes in transcriptional analysis
of the *accBE* operon. B. Physical map of the DNA
fragments cloned in pET22b(+) and used for the

10   heterologous expression of *accB* and/or *accE*. Only the
most relevant restriction sites are shown: B, *Bam*HI;
Bc, *Bcl*I; E, *Eco*RI; K, *Kpn*I; Nd, *Nde*I; N, *Not*I; S,
*Sp*HI.

15   Fig. 2   Attempted disruption of *accB*. A. Diagram showing
the integration of pTR124 through one of the *accBE*
flanking regions and the resolution of the cointegrate
by a second event of homologous recombination. The
crossed out arrow indicates the impossibility of

20   obtaining the replacement of the wild-type *accB* by the
Hyg^R mutant allele. B. The integration of a second
copy of the *accBE* genes in the ΦC31 *att* site of T124
(to yield strain T149) allowed the replacement of the
wild-type *accB* by a mutant allele containing the Hyg

25   resistance cassette.

Fig. 3   Growth-phase dependent expression and
transcription start site of the *accBE* operon. A. S1
nuclease mapping of *accB,* actII-ORF4 and *hrdB,* using

30   RNA isolated from a liquid time course of *S.
coelicolor* M145. Exp, Trans and Stat indicate the
exponential, transition and stationary phase of
growth, respectively. B. The nucleotide sequences of
both strands from the *accB* promoter region are shown.

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The arrow indicates the most likely transcription start point for the *accBE* promoter, as determined by S1 nuclease mapping. The potential -10 and -35 regions for the *accBEp* are underlined. C. S1 nuclease mapping

5   of the *accB accE* intergenic region using a 563 nt probe. FLP represents the full-length RNA-protected fragment that is 13 nt shorter than the probe.

Fig. 4   Growth-phase dependent expression of *accA2* and

10   *accA1*. S1 nuclease mapping of *accA2* (A) and *accA1* (B), using RNA isolated from a liquid time course of *S. coelicolor* M145.

Fig. 5   Mapping of the *accA2* and *accA1* transcription

15   start point. A. High resolution S1 nuclease mapping of the 5'end of the *accA2* transcript. S1, RNA-protected products of the S1 nuclease protection assay. Lanes labelled A, C, G and T indicate a dideoxy sequencing ladder using the same oligonucleotide that was used to

20   make the S1 probe (accA2down). B. High resolution S1 nuclease mapping of the 5' end of the *accA1* transcript. S1, RNA-protected products of the S1 nuclease protection assay. Lanes labelled T, G, C and A indicate a dideoxy sequencing ladder using the same

25   oligonucleotide that was used to make the S1 probe (accA1down). C. Sequence of the *accA2* and *accA1* upstream regions, indicating the most likely transcription start points for the promoters of each of the *accA1* and *accA2* genes (bent arrows). The

30   potential -10 and -35 sequences for the *accA1* and *accA2* promoters are underlined. The potential ribosomal binding sites (rbs) are highlighted with bold letters. The 16 nt direct repeats (DR) found

upstream of the transcription start point of *accAIpl* are indicated with straight arrows.

Fig. 6   Construction and analysis of the *accBE* conditional mutant. A. Diagram showing the integration of pIJ8600 in strain M86 and the expected organisation of the Campbell integration of pTR94 in M94.   Restriction sites: B. *Bam*HI; N, *Not*I; Nd, *Nde*I; S, *Sac*I; Sp, *Sph*I; Xb, *Xba*I. B. Hybridisation analysis of Southern blot of *Sac*I-digested DNAs from M145, M86 and M94.   The probe was the internal *Nde*I-*Xba*I fragment of *accB* shown in A (see Fig. 10).

Fig. 7   Expression of the acyl-CoA components in M86 and M94. A. SDS-PAGE of cell-free extracts of *S. coelicolor* M86 and M94 strains grown in YEME medium containing 10 μg/ml Am with or without the addition of 5 μg/ml Th. B. A duplicate of the SDS-PAGE gel shown in A was subjected to Western blotting and stained for biotinylated proteins by using alkaline phosphatase-streptavidin conjugate.

Fig. 8A   Growth curves of M145, M86 and M94 strains. 10^8 spores of strains M86 and M94 were inoculated in YEME medium containing 10 μg of Am or 10 μg/ml Am and 5 μg/ml of Th. 10^8 spores of M145 were inoculated in YEME. The growth was followed by measuring OD_{450nm}.

Fig. 8B   Actinorhodin production in M94 and M145 in cultures grown in the presence of 5μg of Th.

Fig. 9   Morphological and physiological differentiation of M86 and M94 in the presence of Th.   Spores of M86 and M94 were spread in R2 or R5 medium containing

10μg/ml Am. A drop containing 1 μg of Th was spotted in the centre of each plate. The picture shows the results obtained after the incubation of the plate at 30°C for 48h.

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Fig. 10   The sequence of the amplification product obtained from accB using primers TC16 and TC17. *NdeI* (CATATG) and *XbaI* (TCTAGA) sites introduced into the *accB* by the primers are shown in bold. The 1 kb *NdeI*-*XbaI* fragment was cloned into pIJ8600.

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Fig. 11   A. Amino acid sequences and B. Nucleic acid sequences of *accA1* and *accA2*.

Fig. 12   A. Amino acid sequence and B. Nucleic acid sequence of *accB*.

15

Fig. 13   A. Amino acid sequence and B. Nucleic acid sequence of *accE*.

20

Fig. 14   Plasmid map for the construction of an expression vector for *accA*, *accB* and *accE*.

Example 1:   Cloning of *accBE* genes

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pccB of *S. coelicolor* (Rodríguez and Gramajo, 1999) was used as an heterologous probe in Southern blot experiments. When a *BamHI* digest of *S. coelicolor* DNA was probed with *pccB* and washed under low stringent conditions, a second, low hybridising, band was readily detected (data not shown). The target sequence was cloned from a size-enriched library as a 2.5 kb *BamH1* fragment and sequenced as described in Experimental Procedures (below). The sequence revealed the presence of an incomplete ORF with high

homology to *pccB*. The complete gene was finally cloned as a 6 kb *SstI* fragment yielding pRM08 (Fig. 1). Sequencing and analysis of this DNA fragment revealed the presence of an ORF that exhibited end-to-end similarity with a putative
5   decarboxylase (though the real function is unknown) of *S. cyanogenous* (Westrich et al., 1999), with the *S. coelicolor* PccB (Rodríguez and Gramajo, 1999) and with the β-subunit (PccB) of the *Sac. erythraea* PCCase (Donadio, *et al.*, 1996). The levels of identity were 76%, 57% and 56%,
10 respectively. The gene encoding this new putative carboxyl transferase was called *accB*.

Surprisingly, the sequence also revealed the presence of a small ORF, designated *accE*, whose start codon is only 17 bp
15 downstream of the termination codon of *accB*. A 17 nt inverted repeat, which could function as a factor-independent bidirectional transcriptional terminator (reviewed in Lewin, 1994), separates *accE* from three convergent ORFs with homology to putative proteins of *M.*
20 *tuberculosis* with unknown functions. The putative AccE polypeptide has a deduced molecular mass of 7.07 kDa and no significant homology to this polypeptide was found in a search of sequences deposited in the GenBank database.

25 Upstream of *accB* there is an ORF highly homologous to several known hialuronidases.

Example 2:   *accB* is essential for *S. coelicolor* viability
30
An *accB* mutant was constructed by gene replacement (Fig. 2A). A Hyg-resistant cassette was cloned in the unique *Bam*HI site present in the coding sequence of *accB,* contained in pTR80. After an intermediate construction in

pIJ2925, a *Bgl*II fragment containing the mutated allele was finally cloned in the conjugative vector pSET151. The resulting plasmid, pTR124, was cloned into the *E. coli* donor strain ET12567/pUZ8002 and transferred by conjugation

5   into M145. Exconjugants were selected for Th^R Hyg^R for a simple crossover event. One of the exconjugants, named T124, was taken through four rounds of non-selective growth (SFM Hyg) to promote homologous recombination for the second crossover. Spores were plated to give single

10   colonies and several thousands screened for Th sensitivity (which would have reflected successful gene replacement), but no Th^S isolates were obtained. This result suggested that *accB* is essential for *S. coelicolor* viability.

15   The inventors proposed, however, that if a second copy of *accB* were present in the chromosome of T124, a second crossover event (leading to the replacement of the wild type gene by the Hyg^R mutant allele) would then be allowed. To confirm this hypothesis, pTR149, which contains a copy

20   of the *accBE* genes under its own promoter (see Experimental procedures, Fig. 2B), was first integrated in the ΦC31 *att*B site of T124. (The introduction of a second copy of both genes into the chromosome was prompted by the probability of a polar effect on *accE* taking place after the gene

25   replacement event and because AccE is important for the recovery of a fully active acyl-CoA carboxylase complex – see *in vitro* reconstitution experiments below). The resultant strain T149 (Hyg^R, Th^R, Am^R) was passed through three rounds of sporulation on SFM Hyg Am and after the

30   screening of approximately 500 colonies, 20 were found to be Am^R Hyg^R Th^S. The final chromosomal organization of *accB* in each of the strains constructed (T124, T149 and T149A), was analyzed by Southern blots using an internal fragment of *accB* as a probe.

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Example 3:   **Heterologous expression of** *accB*, *accE* **and in vitro reconstitution of an acyl-CoA carboxylase complex.**

5   Since *accB* proved to be essential for *S. coelicolor* viability, we could not clearly evaluate *in vivo* the physiological function of this gene product.

      In order to study if AccB and AccE were components of an
10   acyl-CoA carboxylase complex, we attempted *in vitro* reconstitution of the enzyme activity by mixing *E. coli* cell-free extracts containing the AccB and AccE with cell-free extracts containing the biotinylated sub-units AccA1 or AccA2. *E. coli* does not contain an ACCase enzyme, so
15   ACCase activity cannot be assayed directly by carboxylation of acetyl-CoA (Polakis *et al.*, 1972); therefore, the acyl-CoA carboxylase activity measured in these crude extracts exclusively represents the activity of the heterologous complexes reconstituted *in vitro*.
20
      Heterologous expression of *accB* and *accE* was attempted by introducing a *Nde*I site at the ATG start codon of *accB*; after an intermediate construction (see Experimental procedures), *accBE* was cloned as a *Nde*I-*Sac*I fragment into
25   pET22(b), yielding pTR88 (Fig. 1). Transformation of *E. coli* BL21(DE3) with this plasmid yielded strain RG8 (Table 1). Crude extracts of RG8, prepared from IPTG-induced cultures, showed a clear over-expression of a 64 kDa protein in a 15 % SDS-PAGE, corresponding to AccB; by
30   contrast, AccE was not clearly visualised by Coomassie blue staining of the same gel (data not shown). *In vitro* reconstitution of an acyl-CoA carboxylase was then attempted my mixing crude extracts prepared from IPTG-induced cultures of RG8 with cell-free extracts of the *E.*

coli strains RG7, which overproduces the biotinylated protein AccA1. After incubation for 1 h at 4 ºC, the mixture was assayed for ACCase and PCCase activity. As shown in Table 2 an enzyme complex showing high levels of

5   both ACCase and PCCase activities was successfully reconstituted

To study if cell-free extracts containing AccB but not AccE were capable of reconstituting an active acyl-CoA

10   carboxylase complex when mixed with cell-free extracts containing AccA1, we constructed a new pET22(b) derivative that only expresses *accB*. For this we took advantage of the *Not*I site present approximately in the middle of the coding sequence of *accE* and cloned the *Nde*I-*Not*I fragment from

15   pTR88 into the expression vector, yielding pTR90 (Fig. 1).

Cell-free extracts of RG9, obtained by transformation of BL21(DE3) with pTR90, showed high levels of soluble AccB after IPTG induction. However, the acyl-CoA carboxylase

20   complex reconstituted *in vitro*, after mixing cell-free extracts of RG9 (AccB) and RG7 (AccA1), showed much lower levels (approximately 10%) of ACCase and PCCase activities than the acyl-CoA carboxylase previously obtained by mixing RG8 with RG7 cell-free extracts (Table 2). Since the levels

25   of AccB in cell-free extracts of RG8 and RG9 were essentially the same, we inferred from these experiments that AccE was necessary in order to obtain a fully active acyl-CoA carboxylase complex.

30   To confirm that the absence of AccE was the responsible of the lower acyl-CoA carboxylase activities, we studied the effect that the addition of cell-free extract containing AccE, had on the crude extracts containing AccB and AccA1 proteins. For this we first constructed strain RG10

(BL21(DE3) containing pTR107) that expresses high levels of soluble AccE (data not shown).

When cell-free extracts of RG10 where mixed with those of RG9 (AccB) and RG7 (AccA1) and incubated for 1h on ice, the levels of enzyme activity where at least five times higher than in the control experiment, without the addition of AccE (Table 2). Although the results presented in this section clearly show that AccE is a functional part of the acyl-CoA carboxylase, enzyme kinetics studies with purified components will be necessary to understand more precisely the role of this protein in the enzyme complex activity. Similar results were obtained in all the reconstitution experiments mentioned above when AccA1 was replaced by AccA2 as the biotinylated component of the acyl-CoA carboxylase, indicating that either AccA1 or AccA2 can be efficiently used as the α-subunit of this enzyme complex.

Example 4:   **Transcriptional analysis of *accBE*, *accA1* and *accA2***

At least four combinations that resulted in active carboxylase complexes have been reconstituted by mixing the β-subunits PccB (Rodríguez and Gramajo, 1999) or AccB (this work) with either of the two almost identical α-subunits, AccA1 or AccA2. In any of these complexes the carboxyl transferase subunit seems to dictate the substrate specificity; thus, PccB seems to recognize only propionyl-CoA, while AccB has a broader substrate specificity, which allows the enzyme to recognize either acetyl- or propionyl-CoA. Moreover, a third complex with PCCase activity has also being described in *S. coelicolor* (Bramwell, et *al.*, 1996). These findings show a remarkable overlapping of gene function in *Streptomyces* species. We followed two different

approaches to gain more information on this; one was the
generation of mutants and the second the study of the mRNA
levels of some of these four genes throughout the different
growth stages by using S1 nuclease protection.

5

S. coelicolor A3(2) strain M145 was grown in SMM medium and
RNA extracted at exponential, transition and stationary
phase. S1 nuclease protection of *accB* was performed by
using a 483 bp PCR product, uniquely labelled at the 5'end

10 of the downstream oligo. Transcription of *accB* occurs
primarily during active growth (exponential and transition
phases), while its level of expression decayed
significantly after entering into stationary phase (Fig
3A). The transcripts of the major essential sigma factor

15 *hrdB* and of the pathway-specific activator gene for
acitnorhodin biosynthesis, *actII-ORF4*, were also studied as
positive controls for the RNAs used in these experiments.
As expected from previous results, *hrdB* was expressed
constantly throughout growth (Buttner, M.J., 1990), while

20 *actII-ORF4* had a peak of expression during transition phase
that shut off in stationary phase (Gramajo, et *al.*, 1993).

The RNA-protected fragments found for *accB* corresponded to
a transcription start site 1 bp upstream, or in the

25 adenine, of the most likely translation start site of *accB*.
Upstream of the transcription initiation site we found a
putative -10 and -35 promoter regions with a high consensus
sequences of promoters recognised by the vegetative σ^{hrdB}
(Strohl, 1991) (Fig. 3B).

30

In order to find out if *accB* and *accE* were co-transcribed
as a unique bi-cistronic mRNA, a new 563 bp probe was
obtained by PCR. For this we used a 5'oligo corresponding
to a sequence within the coding region of *accB* and a

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3'oligo corresponding to a sequence within *accE*. The full-length RNA-protected fragment was easily differentiated from the probe-probe re-annealing due to the addition of a 13 nt tail to the 5'oligonucleotide (Experimental

5 Procedures). The results obtained in this experiment clearly showed that *accB* and *accE* were part of the same transcript, confirming that these two genes form a single-copy operon (Fig. 3C). Moreover, the expression of *accBE* during the different growth phases as detected with this

10 new probe followed the same profile as the expression observed with the probe used for *accB*.

The levels of *accA2* and *accA1* mRNA present throughout growth were also studied by S1 protection experiments (Fig.

15 4). The probe used for *accA2* was a 766 bp DNA fragment generated by PCR and uniquely labelled on the 5'end of the oligo corresponding to the sequence within *accA2*. This experiment showed the existence of three mRNA-protected fragments. The growth phase-dependent expression of two of

20 them, *accA2p1* and *accA2p2*, resemble very much that of the *accBE* operon. Thus, a constant and high level of expression occurs during exponential and transition phase (TP), while the transcription shuts down when the cultures reach stationary phase (Fig. 4A).

25

Considering that the nucleotide sequences of *accA1* and *accA2* are identical from the first two nucleotides upstream of the most probable GTG translation start sites down to the end of the probe (Rodríguez and Gramajo, 1999), it is

30 important to note that a fragment of 185 bp of the *accA2* probe could also be protected by the *accA1* mRNA. Since the lowest RNA-protected fragment observed in Fig. 4A shows a different pattern of expression with respect to *accA2p1* and p2, and considering that the size of the band corresponds

28

to a 185 bp fragment, we believe that this band might represent the level of expression of *accA1* (although we cannot rule out the existence of a third promoter for *accA2*, regulated in a different manner).

5

S1 nuclease protection of *accA1* mRNA was performed by using a 563 bp PCR product, uniquely labelled at the 5'end of the downstream oligo, corresponding to a sequence within *accA1*. As shown in Fig. 4B, the expression of this gene occurs

10 from at least three different putative promoters, and all of them showed a clear burst of expression during the first hours of the TP, which rapidly shut down during late TP. This pattern of transcription resembled very much the one observed for the third RNA-protected band found for *accA2*.

15 The transcription starts sites for the *accA2*p1 and p2 were mapped by high resolution S1 mapping (Fig. 5A and B). The transcription start points and the putative −10 and −35 promoter regions of these two promoters are shown in Fig. 5C. A certain degree of homology was found between the −10

20 consensus sequence of *accA2*p1 and p2 and the promoters recognised by the vegetative σ^{hrdB} (Strohl, 1992). High resolution S1 mapping of *accA1* revealed that the transcription start point of the most abundant mRNA species starts 88 bp upstream of the GTG initiation codon of AccA1

25 and the putative −10 regions resemble, in some extent, the consensus sequences of promoters recognised by σ^{hrdB}. Interestingly, two direct repeat (DR) sequences of 16 bp, containing only two mismatches, were found flanking the putative −35 region of *accA1*p1 and the transcription start

30 point of *accA1*p2 (Fig. 5C). These DRs could represent DNA binding sites recognised by a putative regulator. A third putative promoter, *accA1*p3, was also detected in longer exposures and the most probable nucleotide start sites are also indicated in Fig. 5C.

Example 5: *accBE* **genes are essential in the presence of malonate**

5   The presence of MatC and MatB homologues in *S. coelicolor* suggested that this micro-organism was potentially capable of transporting malonate within the cell through the MatC transporter, and then activating malonate to malonyl-CoA with the putative malonyl-CoA synthetase MatB. To test

10   whether *S. coelicolor* was able to utilize malonate as a sole carbon and energy source, we grew *S. coelicolor* in a modified SMM medium with no casamino-acids and containing 0.4 % malonate instead of glucose as a sole carbon source. In this medium *S. coelicolor* M145 was able to grow,

15   indicating that MatC and MatB could be the proteins involved in the transport and activation of malonate to malonyl-CoA, and suggesting that a decarboxylase that could convert malonyl- into acetyl-CoA should also be present in this bacterium, to allow the use of malonate as a carbon

20   and energy source.

This result encouraged us to test whether this route could also be an alternative pathway to provide malonyl-CoA to the cell. To prove this hypothesis we tried to obtain an

25   acyl-CoA carboxylase minus mutant in the presence of malonate. For this we took spores of strain T124 and grew them in liquid MM containing 0.4 % of malonate instead of glucose. After 36 h of growth we sonicated the mycelia and spread them in SFM medium containing 0.4 % of malonate and

30   incubated until sporulation. Spores were collected and treated in the same way one more time. Finally, spores harvested after the second round of sporulation were diluted out, inoculated in SFM malonate to give aprox. 500 colonies per plate and replica plated in SFM medium with or

without Th. After analyzing approximately 5000 isolated colonies, no Th^S were obtained. This result indicates that although malonate can be efficiently used as a sole carbon and energy source, the pathway involved in its catabolism

5   can not fulfill the malonyl-CoA requirements of the cell.

Example 6:   Construction of a strain with the *accBE* operon under the control of a *tipA* promoter

10   As shown above, the *accBE* operon, which encodes the carboxyl-transferase and a previously unidentified ε sub-unit of an acyl-CoA carboxylase, is essential for the viability of *S. coelicolor* A3(2). In order to regulate the expression of this operon and study its effect on the

15   physiology of this microorganism, we constructed a conditional mutant strain where the expression of the *accBE* operon was under the control of the thiostrepton-inducible *tipA* promoter (Murakami, *et al.*, 1989).

20   A 947 bp fragment containing a modified 5'end of the *accB* gene was cloned under the *tipA* promoter in pIJ8600 (Sun et al (1999) supra) to yield pTR93. After removal of the ΦC31 integration components (*att* and *int*) present in pTR93 we obtained pTR94, which was transformed into the *E. coli*

25   strain ET12567/pUZ8002 (MacNeil et al (1992)/Paget et al (1999)). Conjugation of pTR94 into the *S. coelicolor* strain M145 gave several exconjugants Th^R. One of these exconjugants, designated M94, was purified in SFM medium for further analysis. Integration of pTR94 could only take

30   place by Campbell recombination through the *accBE* homologous sequences, and this event should leave a complete copy of the *accBE* operon under the *tipA* promoter (Fig. 6A). To confirm that this event had occurred in M94, we performed Southern blot experiments of DNA samples

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prepared from strains M145, M94 and M86. The last strain
(M86) was obtained by integration of pIJ8600 in the ΦC31
att site of the chromosome and used as the best isogenic
control for M94 (Fig. 6A). As shown in Fig. 6B, a *SacI*
digested DNA from M145 and M86 lights up a unique
hybridisation band of 5.94 kb that contains the *accBE*
operon. DNA from M94, instead, lights up two hybridising
bands corresponding to the expected sizes for the
integration of pTR94 in the *accBE* operon (Fig. 6A and B).

Example 7:   Acyl-CoA carboxylase levels in M94 and M86

Cultures of the conditional *accBE* mutant M94 grew normally
in YEME medium containing 5 μg of Th. Interestingly, in the
absence of the antibiotic, the cultures were still able to
grow, although at much lower rate. This experiment re-
confirms the leakiness of the *tipA* promoter (M. J. Bibb,
personal communication). In order to determine the levels
of the acyl-CoA carboxylase in conditions of induction or
non-induction we carried out the following protocol. YEME
medium containing 10 μg of Am was inoculated with spores of
M94 (or M86) to give and initial $OD_{450}= 0.1$. Cultures were
grown for 12 h at 30 °C and after that time 5 μg of Th was
added to a half of each culture, keeping the other half as
a control. Both flasks were then incubated for additional
24 h at 30 °C. The harvested mycelia were disrupted by
sonication and cell debris removed by centrifugation. Cell-
free extracts were finally analysed by SDS-PAGE and used
for enzyme assays. Fig. 7A shows a 60 kDa protein that is
only induced in cultures of M94 grown in the presence of
Th; the size of this protein corresponded to the molecular
mass of AccB. We were not able to detect an inducible band
corresponding to AccE. The levels of the biotinylated
components (AccA1 or AccA2) of the acyl-CoA carboxylase, in

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each of the cell-free extracts, were analysed by a modified Western Blotting procedure (Fig. 7B). As shown in this figure the levels of AccA1 and/or AccA2 were not modified by presence of Th. However, cell free-extracts of M94 do

5   contain a slightly higher amount of the 65 kDa protein compared to M86.

ACCase and PCCase activities were assayed in cell-free extracts of M94 and M86. The levels of both enzyme

10   activities were similar in cell-free extracts prepared from cultures of M86 grown in the presence or in the absence of Th (Table 3). Cell-free extracts prepared from induced cultures of M94 show instead a remarkable increase in both ACCase (11.5 fold) and PCCase (3.5 fold) activities,

15   compared with the levels found in non-induced cultures of the same strain or in M86. Moreover, if the enzyme levels found in the wild type strain M145 (Rodríguez and Gramajo, 1999) are compared with those found for M94, the increase in ACCase and PCCase levels were still 4- and 2-fold,

20   respectively (Table 3). These results indicate that by overproducing only two (β and ε) of the three sub-units that form the acyl-CoA carboxylase of S. coelicolor we can increase significantly the levels of this enzyme activity.

25   **Example 8:   Influence of the acyl-CoA carboxylase levels in the physiological properties of M94**

Growth curves (Fig 8A) were determined for the conditional mutant M94 and for M86 by inoculating a spore suspension in

30   YEME medium supplemented with 10 µg of Am, with or without the addition of 5 µg of Th. For M145, YEME medium without the addition of any antibiotic was used. M94 supplemented with the inducer (Th) showed a growth rate during exponential phase very similar to M145, judged from the

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slope of the curves. However, the initiation of growth for M94 seems to occur sooner than in M145, reaching the stationary phase earlier than the wild type strain. When the cultures were not supplemented with Th, M94 grew

5   considerably slower, reaching stationary phase several hours latter than in the presence of Th. Also, the final OD reached by M94 in the presence of Th and by M145 were very similar ($OD_{450}= 3$) after 60 h of growth. Cultures of M86 grew very slowly compared with M94 and M145, independently

10   of the presence or not of Th. However, these cultures levelled off at the final OD reached by M145 and M94 after 50 h of growth.

      Actinorhodin and undecylprodigiosin were also quantitated

15   throughout growth. Table 4 shows that antibiotic production was only detected in cultures of M94 grown in the presence of 1 or 5 µg of Th. No antibiotic production was observed in cultures of M145 or M94 without Th, at least until after 60 h of growth. No antibiotic production was detected in

20   M86.

      To determine the effect of Th induction in M86 and M94, 1 µg of the antibiotic was spotted to a confluent lawn of these strains in R2 and R5 medium supplemented with 10 µg

25   of Am. A striking stimulatory effect in both sporulation and antibiotic production was observed in M94 after 48 h. No stimulation of growth or antibiotic production was observed in M86.

30   Fig. 8B shows the stimulatory effect on actinorhodin production in M94 compared to M145 in cultures grown in the presence of 5µg of Th.

Example 9:   **Co-expression of accA, accB and accE in S. coelicolor**

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The *NdeI-XbaI* fragment of pTR154 (Fig. 14) is introduced into pIJ8600 and then transformed into *S. coelicolor* M145 (Fig. 14). Transformants are selected with apramycin and
5   thiostrepton. Overexpression of the three components *accA2*, *accB* and *accE* results in increased ACCase activity and antibiotic production compared to the wild type M145 strain.

35

Discussion

The use of *pccB* (Rodríguez and Gramajo, 1999) as an
heterologous probe, allowed the successful isolation of a
5   chromosomal DNA fragment containing *accB*, a gene encoding
for a putative new carboxyl transferase of *S. coelicolor*.
This predicted function was based on the high percentage of
identity that AccB showed not only to the *S. coelicolor*
PccB, but to several others biochemical and/or genetically
10   characterized carboxyl transferases reported for
actinomycetes, such as the PccB of *Sac. erythraea* (Donadio,
et al., 1996) and to a less extent to the AccD5 of *M.
tuberculosis* (Cole, *et al.*, 1998) and PccB of *M. leprae*
(Doukhan, 1995). An interesting finding from the analysis
15   of the cloned sequence was the presence of a very small
ORF, named *accE*, immediately downstream of *accB*.

The successful expression of *accB*, *accE* and the BC-BCCP-
(biotin carboxylase- and biotin carboxylase carrier
protein-)encoding genes *accA1* and *accA2* in *E. coli* allowed
20   *in vitro* studies to be performed in order to understand the
role of the corresponding encoded proteins as components of
a previously uncharacterized acyl-CoA carboxylase. The
reconstitution, by mixing cell-free extracts of *E. coli*
25   containing AccB and AccA1 (or AccA2), of an active enzyme
with the ability to carboxylate either acetyl- or
propionyl-CoA clearly established that AccB was the
carboxyl transferase component of an acyl-CoA carboxylase
complex. Interestingly, the small polypeptide, AccE, also
30   showed to play an important role in the reconstitution of a
fully active enzyme complex (Table 2). It remains to be
elucidated whether this protein plays a role as an
allosteric regulator of the enzyme or whether it is a
structural component of the complex. Thus, our results

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represent the first characterization, at both the genetic and biochemical levels, of a prokaryotic acyl-CoA carboxylase.

5   All the acyl-CoA carboxylases studied so far contain the three functional domains in two individual polypeptides (for a review see Brownsey et al., 1997), and none of the purified complexes have shown the presence of a small component equivalent to AccE. Therefore, this might be a

10   distinctive feature for Streptomyces sp. In addition, no AccE homologues have been found in any of the bacteria genomes sequenced so far, an observation that could also support this hypothesis.

15   Malonyl-CoA is an essential component of all living organisms, since it is the main elongation unit for fatty acid biosynthesis (Brownsey et al., 1997). This primary metabolite is synthesised in most species through the carboxylation of acetyl-CoA by an ACCase (Bloch and Vance,

20   1977). If this was also the case for S. coelicolor and, if AccB was the component of an essential acyl-CoA carboxylase, mutation of this gene should be lethal for the micro-organism. Replacement of the wild-type accB for the Hyg^R mutant allele prove to be unsuccessful, and it only

25   occurred when a second copy of the accBE genes was present in the chromosome (Fig. 2B).

These experiments clearly indicated that at least accB was essential for S. coelicolor viability. The fact that both

30   AccA2 (Rodríguez and Gramajo, 1999) and AccB have proved to be essential,.along with the fact that acyl-CoA carboxylase reconstituted in vitro with these two sub-units has the ability to recognise either acetyl- or propionyl-CoA as substrates, strongly suggests that AccA2 and AccB are the α

37

and β components of an essential acyl-CoA carboxylase, whose main physiological role should be the biosynthesis of malonyl-CoA. The transcriptional levels of *accB* and *accA2* throughout growth (Fig.3A and 4A) also support this
5   interpretation, since both genes are principally transcribed during exponential and transition phase. Moreover, ACCase and PCCase activities also showed the highest and constant levels of activities during exponential and transition phase while in stationary phase
10 the activities were low but readily measurable.

In *S. coelicolor,* besides the obvious need for malonyl-CoA biosynthesis during vegetative growth, there is also a requirement for this metabolite during transition and
15 stationary phase, since at least two secondary metabolites (undecylprodigiosin and actinorhodin) are synthesised during these growth-phases and they both require malonyl-CoA for their biosynthesis. Hence, if the ACCase is the only enzyme that synthesises malonyl-CoA in this bacterium,
20 its presence will be also required during the idiophase.

According to the proposed composition of this enzyme complex and based on the transcriptional studies, we propose that the low level of expression of *accA2* and *accBE*
25 during stationary phase is sufficient to produce enough of the α and β components for an active acyl-CoA carboxylase. From the observation that *accA1* mRNA peaks during transition phase, we propose that enough AccA1 might be present in the cytoplasm to compete with AccA2 as the main
30 α sub-unit of this enzyme complex in the stationary phase. However, no difference in antibiotic production has been found between M145 and the isogenic *accA1* mutant MA4 (Rodríguez and Gramajo, 1999).

We have clearly demonstrated the ability of *S. coelicolor* to efficiently utilize malonate as a sole carbon and energy source. A putative pathway for the utilization of this substrate could involve the *R. trifolii* MatC and MatB

5 homologues which are found in the genome of *S. coelicolor*. The biochemical characterization of MatB in *R. trifolii* demonstrated that this protein is a malonyl-CoA synthetase, which catalyzes the formation of malonyl-CoA directly from malonate and CoA. MatC, instead, has not been

10 characterized biochemically but computer analysis indicate that it is a transmembrane protein that could function as a dicarboxylate (malonate for example) carrier (An and Kim, 1998). If these enzymes were part of the pathway that allows *S. coelicolor* to utilize malonate as a sole carbon

15 source, one could also presume that the malonyl-CoA synthesized by MatB should fulfill the malonyl-CoA requirements of the micro-organism. However, we could not show that under these conditions the essential acyl-CoA carboxylase becomes dispensable.

20

Interestingly, the addition of 0.4% malonate to SFM and glucose-MM media produced a clear stimulation of actinorhodin production (data not shown). From this we propose that higher levels of malonyl-CoA were probably

25 available under this growth conditions. From this, and the observation that even the limited levels of the ACCase activity found during the stationary phase of growth of this bacterium are sufficient to allowed regular levels of antibiotic production, the inventors propose that

30 increasing the expression of the ACCase components will probably lead to an improved production of antibiotics.

Experimental Procedures

Bacterial strains, cultures and transformation conditions

S. coelicolor A3(2) strain M145 (SCP1⁻ SCP2⁻) was manipulated as described by Hopwood *et al.* (1985). The strain was grown on various agar media - SFM (Rodríguez and
5   Gramajo, 1999), R2 and R5 - or in 50 ml SMM or YEME liquid media (Hopwood *et al* (1985) supra). *Escherichia coli* strain DH5α (Hanahan 1983) was used for routine subcloning and was transformed according to Sambrook *et al.* (1989). Transformants were selected on media supplemented with the
10   appropiate antibiotics : ampicillin (Ap) 100 µg/ml; apramaycin (Am) 100 µg/ml; chloramphenicol (Cm) 25 µg/ml or kanamycin (Km) 30 µg/ml. Strain BL21(DE3) is an *E. coli* B strain [F⁻ *ompT* (r_B^- m_B^-) (DE3)] lysogenized with lDE3, a prophage that expresses the T7 RNA polymerase downstream of
15   the IPTG-inducible *lac*UV5 promoter (Studier & Moffat, 1986). ET12567/pUZ8002 (MacNeil et al (1992)/Paget et al (1999)) was used for *E. coli* - *S. coelicolor* conjugation experiments (Bierman, 1992). For selection of *Streptomyces* transformants and exconjugants, media were overlayed with
20   thiostrepton (Th) (300 µg per plate), hygromycin (Hyg) (1 mg per plate) or apramycin (Am) (1 mg per plate). Strains and recombinant plasmids are listed in Table 1.

Growth conditions, protein expression and preparation of
25   cell-free extracts
S. coelicolor M145 was grown at 30°C in shake flasks in YEME medium for 24-48 h. When necessary, 10 mg Am ml⁻¹ or 5 mg Th ml⁻¹ were added to the medium. Mycelia were harvested by centrifugation at 5000 x *g* for 10 min at 4 °C, washed in
30   100 mM potassium phosphate buffer pH 8 containing 0.1 mM DTT, 1 mM EDTA, 1 mM PMSF and 10% glycerol (buffer A) and resuspended in 1 ml of the same buffer. The cells were disrupted by sonic treatment (4 or 5 s bursts) using a VibraCell Ultrasonic Processor (Sonics & Materials, Inc.).

Cell debris was removed by centrifugation and the supernatant used as cell-free extract. For the expression of heterologous proteins, *E. coli* strain BL21(DE3) harbouring the appropriate plasmids were grown at 37°C in

5 shake flasks in LB medium in the presence of 25 µg Cm ml^{-1} or 100 µg Ap ml^{-1} for plasmid maintenance. For the expression of biotinylated proteins, 10 µM d-biotin was supplemented to the medium. Overnight cultures were diluted 1:10 in fresh medium and grown to A_{600} 0.4-0.5 before the

10 addition of IPTG to a final concentration of 0.1 mM. Induction was allowed to proceed for 4 h. The cells were then harvested, washed and resuspended in 1 ml buffer A. Cell-free extracts were prepared as described above.

15 Protein methods
Cell-free extracts were analysed by denaturing (SDS)-PAGE (Laemmli, 1970) using the Bio Rad mini-gel apparatus. The final acrylamide monomer concentration was 12% (w/v) for the separating gel and 5% for the stacking gel. Coomassie

20 brilliant blue was used to stain protein bands. The biotinylated proteins were detected by a modification of the Western blotting procedure described by Nikolau *et al.* (1985). After electrophoretic separation, proteins were electro-blotted onto nitrocellulose membranes (Bio-Rad) and

25 probed with alkaline phosphatase-streptavidin conjugate (Bio-Rad) diluted 1:10000. Protein content was determined by the method of Bradford (1976) with BSA as standard.

In vitro reconstitution and assay of the acyl-CoA

30 carboxylase complex
In vitro reconstitution of the enzyme complex was carried out by mixing 100 µg of each of the cell-free extracts shown in Table 2 in a final volume of 300 µl. When AccE was

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not included in the incubation mix, 100 µg of BSA were added instead. The mixes were incubated for 1 h at 4 °C and 100 µg of each used for enzyme assay.

5   ACCase and PCCase activities in cell-free extracts were measured following the incorporation of $H^{14}CO_3^-$ into acid non-volatile material (Huanaiti & Kolattukudy, 1982; Bramwell *et al.*, 1996). The reaction mixture contained 100 mM potassium phosphate pH 8.0, 300 µg BSA, 3 mM ATP, 5 mM

10   $MgCl_2$, 50 mM $NaH^{14}CO_3$ [specific activity 200 µCi mmol^{-1} (740 kBq mmol^{-1})], 1 mM substrate (acetyl-CoA or propionyl-CoA) and 100 µg cell-free protein extract in a total reaction volume of 100 µl. The reaction was initiated by the addition of $NaH^{14}CO_3$, allowed to proceed at 30 °C for 15 min

15   and stopped with 200 µl 6 M HCl. The contents of the tubes were then evaporated to dryness at 95 °C. The residue was resuspended in 100 µl water, 1 ml of Optiphase liquid scintillation (Wallac Oy) was added and ^{14}C radioactivity determined in a Beckman scintillation liquid counter. Non-

20   specific CO_2 fixation by crude extracts was assayed in the absence of substrate. One unit of enzyme activity catalysed the incorporation of 1 µmol ^{14}C into acid-stable products per min.

25   DNA manipulations
Isolation of chromosomal and plasmid DNA, restriction enzyme digestion and agarose gel electrophoresis were carried out by conventional methods (Sambrook *et al.*, 1989; Hopwood *et al.*, 1985). Southern analyses were performed by

30   using P-labelled probes made by random oligonucleotide priming (Prime-a-gene kit; Promega).

Gene cloning and plasmid construction

The synthetic oligonucleotides TC1, 5'-CAGAATTCAAGCAGCACGCCAAGGGC AAG, and TC2, 5'-CAGAATTCGATGCCGTCGTGCTCCTGGTC, were used to amplify an internal fragment of the *S. coelicolor pccB* gene. The reaction mixture contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1 mM MgCl$_2$, 6% glycerol, 25 µM of each of the four dNTPs, 2.5 U *Taq* DNApolymerase, 20 pmol of each primer and 50 ng of *S. coelicolor* chromosomal DNA in a final volume of 100 µl. Samples were subjected to 30 cycles of denaturation (95°C, 30 s), annealing (65°C, 30 s) and extension (72°C, 1 min). A 1 kb PCR fragment was used as a ^{32}P-labelled probe to screen a size-enriched library. A 2.7 kb *Bam*HI fragment containing an incomplete *accB* gene was cloned in *Bam*HI-cleaved pBluescript SK(+), yielding pTR62.

The synthetic oligonucleotide TC16 (5'-TATTCTAGACATATGACCGTTTTGGATGAGG, used to introduce an *Nde*I site at the translational start codon of the *S. coelicolor accB* gene) and TC17 (5'-ACCTCTAGACAACGCTCGTGGACC, used to introduce an *Xba*I site in the *accB* coding sequence) were used to amplify an internal fragment of *S. coelicolor accB* gene, having the sequence shown in Fig. 10. The reaction mixture was the same as the one indicated above. Samples were subjected to 30 or 35 cycles of denaturation (95°C, 30 s), annealing (65°C, 30 s) and extension (72°C, 1 min). The 1 kb PCR product was digested with *Nde*I and *Xba*I (these sites were introduced in the 5′ ends of the oligos TC16 and TC17 and are shown in bold in Fig. 10) and cloned in *Xba*I-cleaved pBluescript SK(+) in *E. coli* DH5α, yielding pTR82. This plasmid was digested with *BstE*II and *Sac*I, ligated with a *BstE*II-*Sac*I fragment cleaved from pRM08 and introduced by transformation into *E. coli* DH5α, yielding pTR87.

An *NdeI-XbaI* fragment from the plasmid pTR82 was cloned in *NdeI-XbaI*-cleaved pIJ8600 (Sun et al (1999)), yielding pTR93. In order to place the chromosomal copy of *accBE* operon under the *tipA* promoter we removed from pTR93 a

5   *HindIII* fragment containing the *int* gene and *att* of ΦC31, yielding pTR94. Plasmid pTR94 was transformed into strain ET12567/pUZ8002 and transferred by conjugation to *S. coelicolor* M145 (Hopwood et al (1985)).

A *NdeI-SacI* fragment from the plasmid pTR87 was cloned in

10   *NdeI-SacI*-cleaved pET22b(+) (Novagen) (pTR88), thus placing the *accBE* operon under the control of the powerful T7 promoter and ribosome-binding sequences. The synthetic oligonucleotides NaccE, 5'-TTATCTAGACATATGTCCCCTGCCGAC, used to introduce an *NdeI* site at the translational start

15   codon of the *S. coelicolor accE* gene, and CaccE, 5'-ATGAATTCTATGCATCGGGTCAGCGCCAGCTG, were used to amplify the *accE* gene of *S. coelicolor*. The reaction mixture was the same as the one indicated above. Samples were subjected to 35 cycles of denaturation (95°C, 30 s), annealing (65°C, 30

20   s) and extension (72°C, 30 s). The PCR product was cloned using pGEM-T easy vector (Promega) in *E. coli* DH5α, yielding pTR106. A *NdeI-EcoRI* fragment from the plasmid pTR106 was cloned in *NdeI-EcoRI*-cleaved pET22(b) (Novagen) yielding the plasmid pTR107, thus placing the *accE* gene

25   under the control of the powerful T7 promoter and ribosome-binding sequences.

Plasmid pIJ8600 was digested with *BglII* and *EcoRI* and the fragment containing *oriT* RK2, *ori* pUC18, *attP* site, *int* ΦC31 and *aac(3)IV* (Am^R cassette) genes was ligated with a

30   linker containing the following enzymes (Mike Butler personal comunication): *BglII*, *AseI*, *EcoRI*, *BglII*, *NdeI*, *KpnI*, *XbaI*, *PstI*, *HindIII*, *BamHI*, *SstI*, *NotI* and *EcoRI*, yielding pTR141. A 4.0 kb *KpnI* fragment containing the

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complete *accBE* operon from pRM08 was cloned into *KpnI*-cleaved pTR141, yielding pTR149.

For an efficient over-expression in *S. coelicolor* of the three components of the acyl-CoA carboxylase complex of
5   this micro-organism, we carried out the construction of pTR156 through the following steps. First we did a PCR amplification of the chromosomal *accBE* operon using the oligo TC16 (5'-TATTCTAGACATATGACCGTTTTGGATGAGG 3'), to introduce a *NdeI* site at the translation start codon of
10 *accB*, and the oligo C-accE (5'ATG AAT TCT ATG CAT CGG GTC AGC GCC AGC 3') to introduce a *NsiI* restriction site at the 3' end of *accE*. The amplified DNA, was then cloned into pGEM-T (Promega), to give pTR99. To introduce a *NsiI* site upstream of the RBS of *accA2* we amplified this gene using
15 the oligo N-accA2 (5' ATG AAT TCA TGC ATG AGG GAG CCT CAA TCG 3'), for the 5' end and the oligo C-accA2 (5' AGA TCT AGA TCA GTC CTT GAT CTC GC 3') containing a *XbaI* and a *EcoRI* site, for the 3' end of the gene. The amplified DNA was cloned in pGEM-T to give pTR112. The *NdeI-NsiI* DNA
20 fragment from pTR99 and the *NsiI-EcoRI* fragment isolated from pTR112 were finally cloned into pET22(b) (Stratagene), previously digested with *NdeI* and *EcoRI*, to yield pTR154. In order to introduce these genes in *S. coelicolor* we sub-cloned the *NdeI-XbaI* fragment, containing *accBE* and *accA2*,
25 from pTR154 to pIJ8600 digested with the same enzymes to give pTR156. See Fig. 14 for plasmid constructions.

Nucleotide sequencing
The sequence of the *SphI* original fragment was performed
30 from plasmids DNA constructed by subcloning ApaI DNA fragments from pRM08 into pSKBluescribe SK(+). Synthetic oligonucleotides were used to complete the sequence. The nucleotide sequence of the *accBE* region was determined by

dideoxy sequencing (Sanger *et al.*, 1977) using the Promega
TaqTrack sequencing kit and double-stranded DNA templates.
The complete sequence of the 1C2 cosmid, that includes the
*Sph*I fragment harbouring *accBE*, is available from the *S.*
5 *coelicolor* genome sequencing project.

S1 nuclease mapping

For each S1 nuclease reaction, 30 µg of RNA were hybridized
in NaTCA buffer (Murray, 1986); solid NaTCA (Aldrich) was
10 dissolved to 3M in 50mM PIPES (pH 7.0), 5mM EDTA, to about
0.002 pmol (approximately 10^4 cpm) of the following probes.
For *accA2* the synthetic oligonucleotide 5'-
GCTTTGAGGACCTTGGCGATG (accA2down), corresponding to the
sequence within the coding region of *accA2,* was uniquely
15 labelled at the 5' end of the oligonucleotide with [^{32}P]-
ATP using T4 polynucleotide kinase. The labelled oligo was
then used in the PCR reaction with the unlabelled
oligonucleotide (accA2up) 5'-GAAGTACAGGCCGAAGACCAC, which
corresponds to a region upstream of the *accA2* promoter
20 region, to generate a 766 bp probe. For *accA1* the synthetic
oligonucleotide (accA1down) 5'-GCGATTTCGCCACGATTGGCG,
corresponding to the region within the coding region of
accA1, was uniquely labelled with [^{32}P]-ATP using T4
polynucleotide kinase at the 5' end of the oligonucleotide.
25 The accA1down oligo was later used in the PCR reaction with
the unlabelled oligonucleotide (accA1up) 5'-
CCGATATCAGCCCCTGATGAC, which corresponds to a region
upstream of the *accA1* promoter to generate a 563 bp probe.
For *accB* the synthetic oligonucleotide (accBdown) 5'-
30 CGTCAGCTTGCCCTTGGCGTG, corresponding to the region within
the coding region of a*ccB,* was uniquely labelled with
[^{32}P]-ATP using T4 polynucleotide kinase at the 5' end of
the oligonucleotide. accBdown was then used in the PCR
reaction with the unlabelled oligonucleotide (accBup) 5'-

CTACGCTCCGGGTGAGCGAAC, which corresponds to a region
upstream of the accB promoter, to generate a 483 bp probe.
For accBE the synthetic oligonucleotide (accBEdown) 5'-
GGAGGGCCGTGATGGCGGCGACTTCCTCGGG, corresponding to the

5   region within the coding region of accE was uniquely
labelled with [^{32}P]-ATP using T4 polynucleotide kinase at
the 5' end of the oligonucleotide. The accBEdown oligo was
then used in the PCR reaction with the unlabelled
oligonucleotide (accBEup) 5'-

10   GAGGAACTGGTACGCGCGGGCG(GTACAAGCAAGCT), which corresponds to
a region within the coding region of accB (bracketed
oligonucleotides are a tail added to the probe to
differentiate probe reannealing from fully protected DNA-
RNA complexes), to generate a 563 bp probe. Subsequent

15   steps were as described by Strauch et al. (1991).

Determination of actinorhodin

1 ml of whole broth was mixed with 0.5 ml of 3N KOH to give
a final concentration of 1N KOH. The solutions were mixed

20   vigorously and centrifuge at 4000 x g for 5 minutes. The
supernatant was collected and measured at A_{640nm}.
Actinorhodin concentration was calculated using the molar
extinction coefficient (in 1 N KOH) at 640 nm of 25.320
(Bystrykh et al., 1996).

25

Determination of undecylprodigiosin

This was carried out according to the procedure of Hobbs et
al. (1990).

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All the above references are hereby incorporated by reference in their entirety, individually and for all purposes.